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CS: A STUDY OF CARCINOGENICITY

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This was a study of the carcinogenicity of o-chlorobenzilidenemalononitrile (CS) in animals. The question of carcinogenicity arose because CS has been described as an alkylating agent and some alkylating agents are carcinogens. The study showed no significant increase of lung tumors in either A/J strain mice or Sprague-Dawley-Wister rats from exposure to CS to Ct's of 50 and 500 mg min/cu m daily for 20 days (5 days a week for four weeks). Exposure concentrations were about 21 mg/cu m. Total Ct's were, thus, 1,000 and 10,000 mg min/cu m. We can be 95% confident that such a dose would not increase the incidence of tumors by more than 5% if an extremely large

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SUMMARY

This was a study of the carcinogenicity of o-chlorobenzilidenemalononitrile (CS) in animals. The question of carcinogenicity arose because CS has been described as an alkylating agent and some alkylating agents are carcinogens.

The study showed no significant increase of lung tumors in either A/J strain mice or Sprague-Dawley-Wistar rats from exposure to CS to Ct's of 50 and 500 mg min/cu m daily for 20 days (5 days a week for four weeks). Exposure concentrations were about 21 mg/cu m. Total Ct's were, thus, 1,000 and 10,000 mg min/cu m. We can be 95% confident that such a dose would not increase the incidence of tumors by more than 5% if an extremely large sample of animals were dosed. A rioter in the open would not be expected to receive a Ct of greater than 10 mg min/cu m and perhaps 1.0 to 2.5 mg min/cu m on the average.

Chronic exposure to very low concentrations in an industrial situation could be of greater concern.

The data indicate that CS is not a potent carcinogen. In view of the numbers of people who might be exposed, additional work would be required for a more positive statement regarding freedom from carcinogenicity.

PREFACE

The work described in this report was authorized under Project Number 1W562606AD22, Medical Effects of Riot Control Agents.

In conducting the research described in this report the invistigators adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences – National Research Council.

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CONTENTS

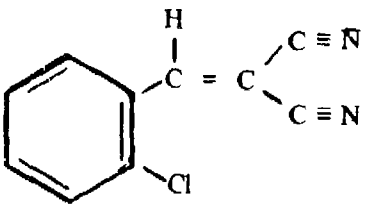
LIST OF TABLES

CS: A STUDY OF CARCINOGENICITY

I. INTRODUCTION.

CS has been described as an alkylating agent.[1] Some alkylating agents are carcinogenic. Therefore, the question arises of the carcinogenicity of CS.

CS, also known as o-chlorobenzylidenemalononitrile and o-chlorobenzalmalononitrile, has the following formula:



II. BACKGROUND.

A. CS as an Alkylating Agent.

It has been stated that CS is probably not an alkylating agent but it can combine with sulfhydryl groups.[2] Binding with nucleic acids has not been reported. Williamson and Witten[3] found that CS binds mainly in the blood. Albumin catalyzes the destruction of CS but the protein is not alkylated. However, CS does react with phosphate and other nucleophilic substances. It has been postulated that most, if not all, carcinogens act through the formation of carbonium ion.[4,5]

In the decomposition of CS by water, a negatively charged anion [$-CH(CN)_2$], but no carbonium ion, is formed.[5,6]

B. CS and Carcinogenicity.

The biological activity of various alkylating agents can be very different. The anticancer alkylating compounds, such as the nitrogen mustards, cause nausea, vomiting, and bone marrow depression. Bone marrow depression is manifested by leucopenia, granulocytopenia, and thrombocytopenia. However, in more than 100 men who were exposed to CS, nausea and vomiting rarely occurred.* No significant changes in the blood picture were noted in these men. Moreover, in

* Aerosol Branch, Medical Research Laboratory, Edgewood Arsenal, Maryland Quarterly Progress Report, January March 1965.

monkeys, dogs, swine, goats, rabbits, rats, and guinea pigs who were exposed once to Ct's ranging from 17,000 mg min/cu m for the swine to 125,000 mg min/cu m for the monkeys, no significant changes were noted in the red blood cell counts, white blood cell counts, hematocrits, prothrombin times, serum glutamic pyruvic transaminase, serum glutamic oxalacetic transaminase, blood urea nitrogen, or creatinine in samples taken 3, 15, and 30 days after exposure.*

In another experiment groups of guinea pigs, dogs, and monkeys were exposed to CS aerosols for 10 consecutive days. Average daily Ct's for two groups of dogs were 4,648 and 18,485 mg min/cu m, respectively. The average daily Ct's for the two groups of guinea pigs were 18,485 and 43,949 mg min/cu m, respectively. The average daily Ct's for two groups of monkeys were 43,993 and 103,225 mg min/cu m, respectively. Blood samples were taken before exposure and 3, 6, 10, 25 and 30 days after the initial exposure. Blood determinations were the same as those on the single inhalation exposure tests. All values throughout the test were normal except those for the white blood cell count. This value increased in dogs and monkeys from low normal levels before exposure to high normal levels following the 3rd, 6th, 10th, 15th and 30th test day after the initial exposure. Antitumor alkylating agents lower the white blood cell count.

Hundreds of tissue sections taken from the animals in the above studies and hundreds taken from animals in other studies have revealed no histopathological indications of carcinogenesis or carcinogenic tendencies.

C. Alkylating Agents and Carcinogenicity.

Alkylation has been described by Ross[7,8] in the following manner. Saturated monovalent alcohols have the general formula of ROH, with R being the alkyl radical. The replacement of a hydrogen atom in a molecule by an alkyl radical is called alkylation. In the process, the alkyl group (R+) becomes attached to an anion or nucleophilic group (X-). The term alkylation is also applied to the addition of an alkyl radical to amines (forming quaternary ammonium ions) and to negatively charged acids (forming esters). The main types of nucleophilic centers encountered in biological material are organic and inorganic anions, amino groups, and sulfide groups. These reactions form esters, alkylated amines, ethers, thioethers, and ammonium and sulfonium compounds.

Various types of alkylating agents are known. A discussion of the following alkylating agents is given in the Encyclopedia of Chemical Toxicology, Volume I, II and XIII:[9-11]

(1) Methanol, ethanol, etc.[9]

(2) Alkyl halides (methyl chloride, ethyl chloride).[9]

(3) Alkyl sulfates and other esters, including alkyl phosphates, such as tricresyl phosphate used in gasoline.[9]

* Aerosol Branch, Medical Research Laboratory, Edgewood Arsenal, Maryland Quarterly Progress Report, January-March 1965.

(4) Alkyl quaternary ammonium derivatives used for germicidal purposes, instrument sterilization, post-operative skin sterilization, wound and tissue irrigation, disinfection of eating utensils, pharmaceuticals (tubocurarine, ganglionic blocking agents, antispasmodics), shampoos, and water-proofers.[10]

(5) Surface-active compounds, heavy-duty household detergents such as Nacconol NR and Santomerse, Ultrawet, and Oronite.[11]

Phenoxybenzamine hydrochloride,[12] (or Dibenzyline,* hydrochloride) is administered clinically to alleviate certain hypertensive conditions. It is believed to combine with smooth-muscle adrenergic excitatory receptors through alkylation. Dibenamine** hydrochloride is another beta-haloalkamine which is used medically as an adrenergic blocking agent.

Ethylenimine and polyethylenimine are used industrially to impart wet strength and abrasion resistance to paper, and to decrease the tendency of rayon and cotton to swell in water. Triaziridinylphosphine oxide makes textiles fire-resistant. This compound, under the name of TEPA, is also used as a sterilant for male insects. Tetramine, the 2,4,6-triaziridinyl derivative of 1,3,5-triazine, is also used as a sterilant.[13] Maleic hydrazide is used as a plant growth regulator.[14]

Since the discovery of the antineoplastic effects of nitrogen mustard in Hodgkin's disease, numerous alkylating agents have been synthesized as antitumor agents. Mechlorethamine (HN_2), chlorambucil (Leukeran), triethylene thiophosphoramide (thiotepa), L-sarcolysin (melphalan), cyclophosphamide (cytoxan), busulfan (myleran) have had widespread use in the clinic. In addition to their therapeutic action, all produce toxic effects on rapidly dividing cells, such as bone marrow and the gastrointestinal tract. Toxic hematopoietic actions are usually shown by decreases in granulocytes and platelets. Nausea and vomiting are produced by some of these substances.[15]

According to Karnofsky and Clarkson[16] many of the anticancer alkylating agents are mutagenic[17,18] and carcinogenic,[19] and preferentially inactivate DNA-containing viruses.[20] The effect of mustards on cells *in vivo*, as well as *in vitro*, consisted of nuclear changes with pycnosis or nuclear enlargement, fragmentation of chromosomes, and inhibition of mitosis.

Carcinogenicity has been shown for some, but not all, alkylating agents. This opprobrium has not attached to the adrenergic blocking compounds Dibenzyline or Dibenamine, tubocurarine, alcohol, or to many of the alkylating agents mentioned above.

Several alkylbenzenesulfonates (alkylating agents used in synthetic detergent products) were found to be noncarcinogenic on the skin and subcutaneously in mice, and on the skin of rabbits.[21]

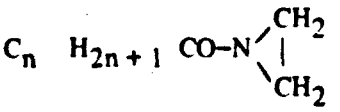
Hartwell[22] and Visser and Ten Seldam[23] applied chloroacetophenone or brombenzyl

* The systematic name of phenoxybenzamine or of Dibenzyline is N-(2-chloroethyl)-N-(1-methyl-2-phenoxyethyl)benzylamine.

** Dibenamine is a registered trade name. It is N-(2-chloroethyl)dibenzylamine.

cyanide in acetone twice weekly to the skin of mice for 5 months. No tumors were found.

Ethylenimine is carcinogenic.[19] Monofunctional acylethylenimines of the sample fatty acid type:



are carcinogenic, but alkanosulfonylethylenimines (also acyl derivatives)are not.[19,24]

That the industrial use of alkylating agents on paper, rayon, and cotton has been associated with carcinogenicity is questionable.

B-CME [bis(chloromethyl)ether, $Cl-CH_2-O-CH_2-Cl$] is a potent alkylating carcinogen for mouse skin, whereas CMME (chloromethyl methyl ether, $Cl-CH_2-O-CH_3$) is inactive.[25]

N-Nitrosomethylamine is recognized as a carcinogen. The N-Nitrosodiphenylamine appears to be noncarcinogenic and has been adequately tested.[26,27]

Maleic hydrazide[28] produces chromatid breaks[29,30,31] and is mutagenic.[32,33] An attempt to demonstrate carcinogenicity was unsuccessful.

It is difficult to relate chemical structure and carcinogenicity for any type of chemical. An outstanding example of this is shown by two non-alkylating substances. β-naphthylamine induces tumors in the urinary bladder in dogs[34] and hepatomas in mice.[35,36,37] The etiological role of β-naphthylamine in bladder cancer in dye workers has been established.[34,38,39,40] In contrast, α-naphthylamine does not cause hepatomas in mice or bladder tumors in dogs.[38,39,40]

III. EXPERIMENTAL.

A. Procedures.

In our studies, groups of 100 (50 males and 50 females) A/J strain mice (a tumor-sensitive strain[4]) and Sprague-Dawley-Wister rats were exposed 5 days per week for 4 weeks to aerosolized CS in methylene chloride at daily Ct's of 50 or 500 mg min/cu m; to urethane in methylene chloride at a daily Ct of 500 mg min/cu m; or to methylene chloride alone at a daily Ct of 500 mg min/cu m. Groups of 100 mice and 100 rats were used as unexposed controls.

The average daily concentrations and exposure times for CS were 21 (13-35) mg/cu m for 2.5 (1.8-3.0) minutes and 21 (13-35) mg/cu m for 25 (18-30) minutes. The average daily

concentrations and exposure times for urethane were 21 (12-37) mg/cu m and 21 (20-30) minutes. The airborne concentrations were measured analytically. The daily and accumulated Ct's are shown in table I.

The methylene chloride concentration was produced nominally by dispersing as much solvent (by weight) as CS (high dose) and urethane.

All animals were observed daily and weighed at weekly intervals for 84 days. After that time, all animals were housed for continued observation. At 6, 12, 18, and 24 months, representative groups were sacrificed and examined for tumors grossly and microscopically.

B. Results.

The weight gains in the control and exposed mice were similar (table II). The weight gains of control rats (table III) were slightly and perhaps insignificantly greater than those in the experimental groups.

A total of 14 experimental mice and one control mouse, and 10 experimental rats and one control rat died during the 4 weeks of exposure (table IV and V).

Table VI shows the frequency of pulmonary tumors. Analysis of the data by Student's *t* test indicates that the tumor frequency in the urethane group of mice was significantly higher at the 0.05 level than frequency in the controls and the group that received methylene chloride. The frequencies in all other groups of mice did not differ from the controls. There was a possible difference between those that received methylene chloride and the mice that received the low dose of CS. There was no difference between the methylene chloride group and the mice given the high dose of CS.

In the rats, there were no statistical differences between the controls and any group, or between the methylene chloride group and any other group.

Pulmonary tumors were found in 3/95 rats that has received the high dose of CS. Pulmonary tumors were found in 1/86 control rats. Fourteen tumors in the subcutaneous tissues were found in the group sacrificed at 18 months. Seven of the tumors were in control animals and none were in CS-exposed animals. The control rats sacrificed at 18 months also had two tumors in the liver, one in the kidney, and one in the spleen. One uterine tumor was found in a CS-exposed rat at this time period. Consequently, 3/95 pulmonary tumors in the high-dose CS group cannot be considered as significant or attributable to the compound.

At 24 months, seven of the eight A/J mice exposed to the low dose of CS had pulmonary tumors. However, they do not seem to be attributable to CS because five of the 12 in the methylene chloride group also had pulmonary tumors and none of four exposed to the high dose of CS had such tumors.

Table I. Exposure Dosages of CS for Mice and Rats

Day of exp.	Exposure Ct value					
	Low CS*		High CS*		Urethane**	
	Daily	Cumulative	Daily	Cumulative	Daily	Cumulative
	Mg min/cu m					
1	40	40	400	400	450	450
2	77	117	770	1170	630	1080
3	56	173	560	1730	525	1605
4	40	213	400	2130	360	1965
5	60	273	600	2730	920	2885
6	36	309	360	3090	750	3635
7	51	360	510	3600	560	4195
8	39	399	390	3990	380	4575
9	40	439	400	4390	400	4975
10	48	487	480	4870	420	5395
11	51	538	510	5380	420	5815
12	69	607	690	6070	360	6175
13	45	652	450	6520	460	6635
14	51	703	510	7030	400	7035
15	70	773	700	7730	450	7485
16	48	821	480	8210	450	7935
17	42	863	420	8630	525	8460
18	45	908	450	9080	500	8960
19	58	966	580	9660	600	9560
20	50	1016	500	10160	480	10040
AVG.	51		508		502	

* The average CS concentration was 21 (13-35) mg/cu m. Exposure times for low CS exposure averaged 2.5 (1.8-3.0) minutes. Exposure times for high CS exposures averaged 25 (18-30) minutes.

** The average urethane (ethyl carbamate reagent grade, Fisher Scientific Co.) concentration was 21 (12-37) mg/cu m. Average exposure time was 21 (20-30) minutes.

Table II. Comparative Mouse Weights (Both Sexes)

No. of days	Average weight per mouse				
	Unexposed control	High CS	Low Cs	Urethane	Solvent control ($MeCl_2$)
	gm				
	Exposure				
0					
7					
14	19.0	18.5	18.7	19.6	19.2
21	19.5	19.2	19.8	20.9	20.1
28*	21.2	21.0	20.6	21.6	21.6
	Recovery				
35	21.7	21.0	21.2	22.7	21.8
42	21.6	21.4	21.5	21.6	21.8
49	22.1	20.4	22.2	22.4	22.4
63	22.7	21.4	22.7	23.0	23.1
84	23.7	23.0	22.9	23.8	23.4

* Last day of 4-week exposure period.

Table III. Comparative Rat Weights (Both Sexes)

No. of days*	Average weight per rat				
	Unexposed control	High CS	Low CS	Urethane	Solvent control ($MeCl_2$)
	gm				
	Exposure				
0	68	67	69	60	69
7	107	88	89	87	89
14	146	118	116	108	116
21	179	149	147	134	146
28**	209	177	176	159	177
	Recovery				
35	235	206	210	209	214
42	254	233	231	229	237
49	269	249	246	246	251
63	292	278	271	268	278
84	318	302	298	297	303

* Rats were 25 ± 1 days old at start.
** Last day of exposure period.

Table IV. Mouse Deaths

Agent	Deaths				
	During exposure period				Post-exposure
	1st week	2nd week	3rd week	4th week	No. of days
Control		1			
High CS	5	2			
Low CS	1				
Urethane	4				
$MeCl_2$	2				
TOTALS	12	3	0	0	0

Table V. Rat Deaths

Agent	Deaths				
	During exposure period				Post-exposure
	1st week	2nd week	3rd week	4th week	
Control					1 at 5 days
High CS	3				
Low CS	2	1			
Urethane	2				
$MeCl_2$				1	1 at 40 days
TOTALS	7	1	0	1	2

Table VI. Pulmonary Tumors in Animals Following 20 Inhalation Exposures to Various Chemicals or Agents

Agent or chemicals	Average Daily Ct	Pulmonary tumors – A/J mice					Pulmonary tumors – A/J rats				
		6 months	12 months	18 months[a]	24 months	Total	6 months	12 months	18 months[b]	24 months	Total
	mg min/cu m										
Controls	–	2/24	0/25	15/25	2/7	19/81	0/26	0/23	1/25	0/12	1/86
$MeCl_2$	500	2/24	2/23	4/25	5/12	13/84	0/24	0/26	0/25	0/20	0/95
Urethane	500	5/24	7/25	16/29	3/3	31/81	0/23	0/25[c]	0/26	0/17	0/91
CS	50	0/24	3/25	14/25	7/8	24/82	0/23	0/24[d]	0/25	0/17	0/89
CS	500	2/22	5/25	11/25	0/4	18/76	0/25	1/29	2/25	0/16	3/95

a/ Subcutaneous tumors: (14 found-various types): seven in control animals, none in CS-exposed animals.
Internal organ tumors: One CS-exposed rat had a uterine tumor.
Four tumors were found in control animals - two in liver, one in kidney, and one in the spleen.

b/ Other tumors: (1) Mammary gland
2-Adenoma
10-Fibroadenomas
3-Adenocarcinoma
1-Fibroma
(2) Inclusion cyst – skin

c/ One rat of this group had a fibroadenoma in the inguinal region.

d/ One rat of this group had an ovarian granulosa cell tumor.

IV. DISCUSSION.

The A/J strain mice were chosen as tumor sensitive.[41] Urethane was included in the study as a positive control since it causes tumors when given intraperitoneally in doses of 1 to 1.5 mg/gm.[41-44] In the present study the inhalation of urethane produces an incidence of pulmonary (31/81 = 38%) in the A/J mice which was significantly greater than that of the controls (19/81 - 22%). The tumor incidences in the mice which received methylene chloride (13/84 = 15%), the low Ct of CS (24/82 = 29%), or the high Ct of CS (18/76 = 24%) were not statistically different from the controls. There was no significant tumorigenic activity in any of the various groups of rats.

The reliability of the test method requires some comment. The results must be related to the number of animals used. Tumor incidence in control A/J mice was 22%. Based on the number of animals used there was no statistical difference between an incidence of 22% and 29% (low dose CS). There was a statistical difference between 29% and 38% (urethane). Thus, a tumor incidence of 9% (38%-29%) might be induced by a compound and not be detected by the test method. To reduce this unmeasured area (9%) would require the use of large numbers of animals. No test is a guarantee that a given compound will not cause tumors in any individual in a population of infinite size.

V. CONCLUSION.

CS was not tumorigenic to A/J-strain mice and Sprague-Dawley-Wistar rats which were exposed 5 days/week for 4 weeks to aerosols at daily Ct's 0f 50 or 500 mg min/cu m. CS in concentrations of 1.0 mg/cu m is sufficiently irritating to be unpleasant or intolerable to most people in a minute or less. Consequently, it is not likely that anyone would receive repeated exposure in the magnitude used in these experiments. A rioter in the open would not be expected to receive a Ct greater than 10 mg min/cu m and perhaps 1.0 to 2.5 mg min/cu m on the average. Chronic exposure to very low concentrations in an industrial situation could be of greater concern.

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